PATENT APPLICATION

Our Docket No. 20050022.ORI

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re App	:	Colin M. Casimir	: October 14, 2009
S.N.	:	10/520,745	: Art Unit 1632
Filed	:	August 22, 2005	:Examiner Wu Cheng Winston Shen
For	:	METHODS OF MAKING VIRAL PARTICLES HAVING A MODIFIED CELL BINDING ACTIVITY AND USES THEREOF	

AMENDMENT UNDER 37 CFR 1.111

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I CERTIFY THAT THIS PAPER IS BEING TRANSMITTED VIA THE OFFICE ELECTRONIC FILING SYSTEM ADDRESSED TO: COMMISSIONER FOR PATENTS, P.O. BOX 1450, ALEXANDRIA, VA 22313-1450, ON OCTOBER 14, 2009 (37 CFR 1.8a)

Arthur L Lewis

Sir:

This paper is submitted in response to a non-final Official Action dated April 14, 2009. That Action carried a shortened-statutory period for reply set to expire in three months or on July 14, 2009. This response is being submitted with a Petition of an extension of time of three months, together with the required fee, to extend that date until October 14, 2009.

It is requested that the following amendments, remarks and other documents be entered:

Amendments to the Claims:

The following represents a complete listing of the claims in this application indicating the present status of each, including any amendments sought to be entered at this time. Any claims that have been canceled or withdrawn have been canceled or withdrawn without prejudice or disclaimer of any subject matter therein. The applicant specifically reserves the right to pursue any and all such claims in continuing and/or divisional applications. In this paper, claim 43 has been amended. Claims 43-67 are pending in this application. Claim 46 and previously withdrawn claims 49 and 57-67 are canceled. Thus, claims 43-45, 47-48 and 50-56 remain under consideration.

Listing of Claims

1-42(Canceled).

43(currently amended). A method of making a viral particle having a modified cell binding activity comprising:

(i) providing a viral packaging cell containing viral nucleic acid encoding an enveloped viral particle, wherein said viral particle is enveloped using an envelope unable to naturally bind to cells of a species being targeted, said viral particle having a first cell binding activity wherein the viral packaging cell also contains exogenous nucleic acid encoding a passenger peptide binding moiety designed to modify said first cell binding activity of said viral particle;

(ii) expressing the viral nucleic acid and exogenous nucleic acid encoding the passenger peptide binding moiety so that the passenger peptide binding moiety is provided at a cell membrane of the packaging cell and a viral particle buds from said packaging cell membrane thereby allowing the passenger peptide binding moiety to be incorporated into the viral particle to modify its first cell binding activity, wherein the passenger peptide binding moiety is selected from the group consisting of cell growth factors, antibodies or antigen-binding fragments thereof, moieties that recognize a target cell [--] specific surface antigen, and moieties that are at least a part of a member of a binding pair comprising a target [--] cell specific cell [--] surface receptor and its ligand and wherein said passenger peptide is other than one naturally derived from the virus or said packaging cell.

44(previously presented). A method as in claim 43 wherein the peptide binding moiety is provided at an outer plasma membrane of the cell.

45(previously presented). A method as in claim 43 wherein the viral particle is derived from a retroviral vector.

46(canceled).

47(previously presented). A method as in claim 43 wherein the passenger peptide binding moiety is membrane-bound stem cell factor.

48(previously presented). A method as in claim 43 wherein the viral packaging cell line comprises additional nucleic acid which can be expressed to provide a bioactive agent which is active in or on a target cell.

49(canceled).

50(previously presented). A method as in claim 48 wherein the bioactive agent has a direct or indirect cytotoxic function.

51(previously presented). A method as in claim 50 wherein the bioactive agent is any one selected from the group consisting of ricin; tumour necrosis factor; interleukin-2; interferon-gamma; ribonuclease; deoxyribonuclease; Pseudomonas exotoxin A; and caspase.

52(previously presented). A method as in claim 48 wherein the bioactive agent is an enzyme capable of converting a relatively non-toxic pro-drug into a cytotoxic drug.

53(previously presented). A method as in claim 52 wherein the bioactive agent is either cytosine deaminase or thymidine kinase.

54(previously presented). A method as in claim 43 wherein the modified cell binding activity allows the viral particle to bind to a target cell.

55(previously presented). A method as in claim 54 wherein the target cell is selected from the group consisting of mammalian cells, human cells, quiescent cells, human haematopoietic stem cells, cancer cells and mammalian T-cells.

56(previously presented). A viral particle having a modified cell binding activity obtainable by a method as in claim 43 wherein the modified cell binding activity is conferred by a peptide other than a chimaeric viral envelope polypeptide.

57-67(canceled).

REMARKS

According to the above amendments, claim 43 has been amended. Claims 43-45, 47-48 and 50-56 remain in this application and are currently being examined. No claim has been allowed.

Claim Rejections Under 35 USC § 112

Claims 43, 45, 47, 48 and 50-56 remain rejected under 35 USC § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. This rejection has several facets.

The Examiner has rejected claim 43 based on the appearance of three "--" signs which leaves interpretation of the language in doubt. Additionally, step (ii) is said to recite a different scope of cells from that recited in step (i).

Accordingly, claim 43 has been amended to remove the "--" signs and make the cells in steps (i) and (ii) consistent. The Examiner is respectfully requested to reconsider and withdraw these rejections.

It is noted that, additionally, independent claim 43 has been rejected as unclear because it fails to relate back to the preambles in the positive process, "a modified cell binding activity" being deemed required by the claimed method. This rejection is respectfully traversed as applicant believes that modifying "its first cell binding activity" in step (ii) of claim

43 does, in fact, require "a modifying cell binding activity". Reconsideration and withdrawal of this rejection is respectfully requested.

In addition, claims 43-45, 47, 48 and 50-56 remain rejected under 35 USC § 112, first paragraph, because the application is deemed enabling only insofar as it relates to the expression of hSCF on a retroviral packaging cell and the targeting of a c-kit-expressing cell. This rejection is also respectfully traversed.

In conjunction with the enablement requirement, the Examiner is asked to revisit applicant's earlier remarks and is asked to consider a Declaration by the inventor, Colin M. Casimir, together with an exhibit evidencing additional experimental data which is being submitted herewith. Applicant believes that the data demonstrates that proteins other than hSCF can be successfully used and that this result is not speculative and has been demonstrated without the need for "undue" experimentation.

In addition to the Declaration and accompanying data, the Examiner is asked to consider also the work of Baltimore and Wang (PNAS, 103(31):11479-11484, 2006), attached hereto as Exhibit A, which, applicant believes, has shown that other skilled practitioners in the art have clearly been able to readily pick up on the work described in the present specification and adopt it to their own needs. They have substituted ecotropic envelope with a mutant form of spleen necrosis virus envelope (not a retrovirus incidentally) to make targetable lentiviruses. They

were successful in this with SCF (though this has just only just been published), but have additionally used CD20 and antibody for specific targeting. One aspect that these authors have picked up on, which is an idea first raised by the present inventor in a paper in *Blood* in 2004 (Chandrashekran et al, 104(9):2697-2703, 2004), attached hereto as Exhibit B, is the separation of binding and fusion functions to two separate molecules. Naturally occurring viral envelopes perform both functions in a single molecule.

It is believed that the predictability and usefulness of the claimed invention is being increasingly confirmed by those skilled in the art and it is therefore believed that the present enablement rejection has been met and should be reconsidered and withdrawn.

Claim Rejections Under 35 USC § 102

Claims 43-45, 54 and 55 are rejected under 35 USC § 102(a) as being anticipated by Gollan et al, *J Virol.* 76(7):3558-63, 2002). This rejection is respectfully traversed.

Gollan et al is not believed to provide any teaching that represents a rational or predictable approach to cell modification. It should be noted that they appear to make insertions at random in the envelope and thereafter test for any changed properties, rather than presenting a systematic approach. Most of the modifications they make inactivate the envelope protein, or compromise it in some way. They have no way of

predicting the outcome of the modifications until they are tested and the method requires the experimenter to make a very large number of modifications to pick up any with altered properties. This approach cannot be used to make changes of a predictable nature and is therefore of limited applicability, especially as it is clear that none of the envelope modifications can be made without some compromise to its function and its functional efficiency. Thus, Gollan et al is not seen to anticipate any of applicant's claims and reconsideration and withdrawal of this rejection is respectfully requested.

Claims 43-45, 47 and 54-56 are also rejected under 35 USC § 102(b) as being anticipated by Povey et al (*Blood*, 92(11):4080-9, 1998) as evidenced by Hammarstedt et al, cited by the applicant. This rejection is also respectfully traversed. Hammarstedt et al only relates to membrane proteins that are endogenously expressed cellular components. Therefore, it cannot be predicted from this paper that expression of extra-genomic material will occur in such a way that it can be guaranteed that it will become incorporated in the virus that buds from such a modified cell. Although there is no reason to assume that such a protein will behave differently to an endogenous one but equally one cannot be sure of this either.

Applicant strongly believes that Povey et al cannot be used in the context that the Examiner is using it. The modification made in that reference is made solely to change the properties of

the packaging cell and not the viruses that emanate from it. There is simply nothing here that says one way or the other whether the SCF on the packaging cells is incorporated into the virus. The effects on transduction of target cell can be wholly explained by the effects on the quiescence of the target cells and the increased binding through virus/packaging cell interactions. In these experiments virus was never delivered to target cells as a purified virus preparation but only via co-culture of retroviral producer cells and target cells. Thus, it is impossible to attribute any properties to the virus itself, independent of the cell-cell interactions. In short, Povey et al does not tell you whether the surface SCF is incorporated into virions budding from this cell line and even were it to do so, it clearly tells you nothing about the biological activity in such circumstance, or whether they could in any way influence viral/host cell interaction e.g. through binding or directing viral entry.

Claim Rejections Under 35 USC § 103

Claims 43, 48, 50 and 51 are rejected under 35 USC § 103(a) as being unpatentable over Povey et al (above) in view of Hammarstedt et al (above) and Dropulic et al (USPN 6,114,141). This rejection is also respectfully traversed.

It is believed that applicant has adequately demonstrated significant differences between any combination of Hammarstedt et al with Povey et al with regard to the present claims and it is

further believed that Dropulic et al does not introduce any teaching or suggestion that overcomes the deficiency of those references and, therefore, applicant believes his claims to patentably distinguish over this combination and, this being the case, he requests that the Examiner reconsider and withdraw this rejection.

The same conclusion can adequately be applied to the rejection of claims 43, 48, 52 and 53 under 35 USC § 103(a) based on Povey et al (above), Hammarstedt et al (above) and Guber et al (USPN 5,691,177), previously cited. This combination also fails to suggest or teach anything that overcomes what is believed to be a clear inventive step between the claims of the present application and the cited combination. Accordingly, reconsideration and withdrawal of this rejection is also respectfully requested.

In general, it appears to be the Examiner's view that it is impossible to make rational or predictable changes in viral tropism by expressing a new molecule on the virus surface based on the present disclosure. This is clearly not true as viral tropism can be altered dramatically simply by substituting one envelope for another e.g. ecotropic an amphotropic pseudotyped viruses have totally different host ranges and these are known and consistent. Conversely, in relation to the presently discloses display methodology, it is also established that other non envelope proteins in the viral surface can influence

infectivity as the identity of the packaging cells (species, cell type, etc) can also have an effect. Undoubtedly, it is not impossible for virus to enter cells by envelope/receptor independent mechanisms but this is clearly of interest only to virologists where very rare events can be of significance, say in the case of a pathogenic organism. Consider that if such mechanisms were of any importance to viral tropism, then it would not be possible for the infectivity of ecotropic virus on human cells to be three or more orders of magnitude lower than on mouse cells where its receptor is present.

The problems over charge, folding, post-translational modification are all essentially spurious as in general for display or targeting purposes we are talking about the use of proteins that naturally locate to the membrane and are not modified or altered in any way and being expressed in mammalian cells (generally human) will undergo all these processes in exactly the same way they do in the cells that normally synthesize them. That many proteins can be successfully engineered is also illustrated by the IL-2 data.

Accordingly, in view of the above amendments, taken together with the explanatory remarks herein, the Examiner is respectfully requested to reconsider and withdraw the present rejections and allow the claims.

Should minor issues remain which, in the opinion of the Examiner, could be resolved by telephone interview, the Examiner is invited to contact the undersigned attorney at his convenience to discuss and resolve same.

Respectfully submitted,

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Targeting lentiviral vectors to specific cell types *in vivo*

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Contributed by David Baltimore, June 15, 2006

We have developed an efficient method to target lentivirus-mediated gene transduction to a desired cell type. It involves incorporation of antibody and fusogenic protein as two distinct molecules into the lentiviral surface. The fusogen is constructed by modifying viral envelope proteins, so that they lack the ability to bind to their cognate receptor but still retain the ability to trigger pH-dependent membrane fusion. Thus, the specificity of such a lentiviral vector is solely determined by the antibody, which is chosen to recognize a specific surface antigen of the desired cell type. This specific binding then induces endocytosis of the surface antigen, bringing the lentivirus into an endosome. There, the fusogen responds to the low pH environment and mediates membrane fusion, allowing the virus core to enter the cytosol. Using CD20 as a target antigen for human B cells, we have demonstrated that this targeting strategy is effective both *in vitro* and in intact animals. This methodology is flexible and can be extended to other forms of cell type-specific recognition to mediate targeting. The only requirement is that the antibody (or other binding protein) must be endocytosed after interaction with its cell surface-binding determinant.

antibody | gene therapy | lentivirus | retrovirus | targeted gene delivery

Gene therapy is the introduction of a functional gene into a target cell to provide a therapeutic advantage (1). A particularly desirable gene therapy protocol would be to precisely deliver a gene of interest to specific cells or organs *in vivo* by means of administration of a designed gene delivery vehicle. Certain viruses are natural gene delivery systems, and much effort has been focused on engineering viral vectors as gene transfer vehicles (1, 2). Among these vectors, ones derived from oncoretroviruses and lentiviruses exhibit promising features because they have the ability to produce stable transduction, maintain long-term transgene expression and, for lentiviruses, to transduce nondividing cells. Targeting such viruses to particular cell types has proved to be challenging. We report here a general methodology that allows such targeting, even *in vivo*, and that is remarkably flexible.

Many attempts have been made to develop targetable transduction systems by using retroviral and lentiviral vectors (3, 4). Significant effort has been devoted to altering the envelope glycoprotein (Env), the protein that is responsible for binding the virus to cell surface receptors and for mediating entry. The plasticity of the surface domain of Env allows insertion of ligands, peptides and single-chain antibodies (5–14) that can direct the vectors to specific cell types. However, this manipulation adversely affects the fusion domain of Env, resulting in low viral titers. The unknown and delicate coupling mechanisms of binding and fusion make it extremely difficult to reconstitute fusion function once the surface domain of the same molecule has been altered (4). Another approach involves using a ligand protein or antibody as a bridge to attach the virus to specific cells (15–18). The challenge to this approach is that the Env, once complexed with the one end of the bridge molecule, fuses inefficiently. Because no practical strategies are available for targeted *in vivo* gene delivery, current gene therapy clinical trials are based on *in vitro* transduction of purified cells followed by infusion of the modified cells into the patient. This is an expensive procedure, with significant safety challenges.

Our strategy involves uncoupling the target cell recognition function from the fusion function by providing them in separate proteins. For recognition, we use antibodies, and, for fusion, we use a viral glycoprotein that has been mutated to inactivate its binding ability. We make lentiviral vectors that incorporate both molecules into their surface. Our working hypothesis was that the antibody should recognize a molecular constituent on the target cell membrane and attach the lentivirus to the cell surface (Fig. 5, which is published as supporting information on the PNAS web site). Antibody binding should then induce endocytosis, bringing the lentivirus into an endosome. There, the fusogenic molecule (FM) should respond to the low pH environment and trigger membrane fusion, allowing the virus core to enter the cytosol. After reverse transcription and migration of the product to the nucleus, the genome of the vector should integrate into the target cell genome, incorporating the vector's transgene into the cell's inheritance.

Results

Construction of pH-Dependent Fusogen. Effective FMs for the proposed system should be able to incorporate into the lentivirus envelope and induce membrane fusion at low pH, independent of receptor binding. There are two classes of such FMs (19). The class I fusogens trigger membrane fusion using helical coiled-coil structures whereas the class II fusogens trigger fusion with β barrels. These two structures have different mechanics and kinetics (19), and both were evaluated to determine which would be better for the promotion of infection. One class I fusogen, HA from influenza A/fowl plague virus/Rostock/34 (FPV), was previously found to pseudotype murine leukemia virus (MLV) (20). Cannon and coworkers (21) created a binding defective version of FPV HA designated as HAmu (Fig. 1*A*). When incorporated into MLV displaying a functionally attenuated envelope glycoprotein, HAmu could enhance viral transduction efficiency (21). HAmu-mediated fusion is thought to be independent of receptor binding (3). The class II FM that we tested was the Sindbis virus glycoprotein from the alphavirus family (22) and is designated as SIN. SIN consists of two transmembrane proteins (23), one responsible for fusion (E1) and the other for cell binding (E2). SIN is known to pseudotype both oncoretroviruses and lentiviruses. By inserting the IgG-binding domain of protein A (ZZ domain) into the E2 protein and making several additional mutations to inactivate the receptor-binding sites, Chen and coworkers (16) made a binding-deficient and fusion-competent SIN. We adapted this form of SIN but replaced the ZZ domain with a 10-residue tag sequence, for which

Conflict of interest statement: No conflicts declared.

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Abbreviations: FM, fusogenic molecule; Env, envelope glycoprotein; SIN, Sindbis virus glycoprotein; αCD20, anti-human CD20 antibody; TU, transduction units; PBMC, peripheral blood mononuclear cells; FPV, influenza A/fowl plague virus/Rostock/34.

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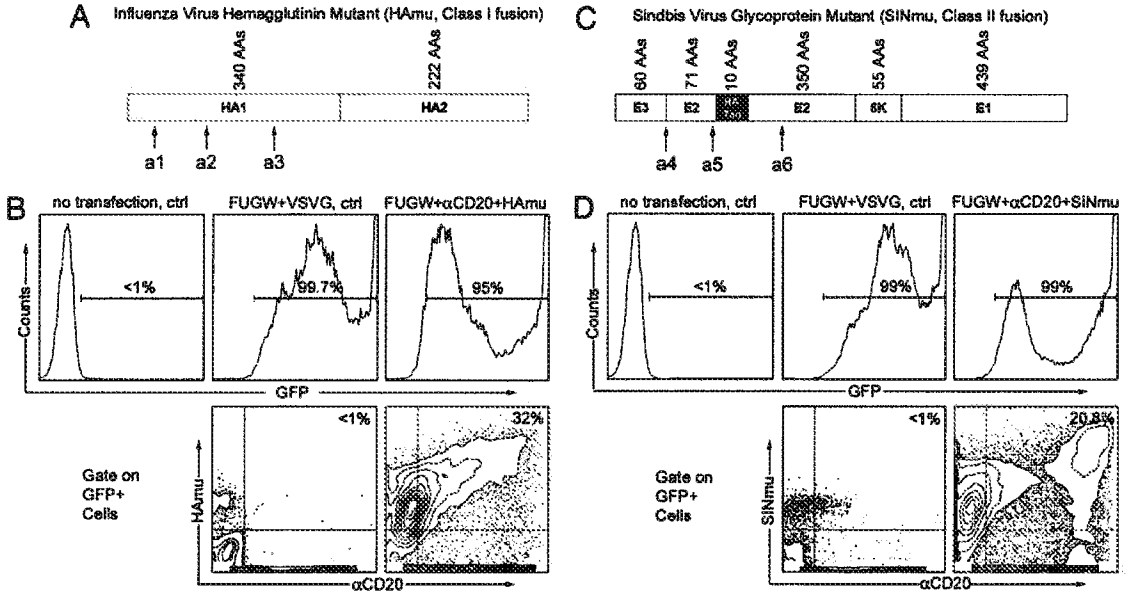

Fig. 1. Coexpression of antibody and fusogenic protein on the surface of the virus packaging cell line. (*A*) The class I fusion protein HAmu derived from influenza A (FPV) HA. HA contains two glycoproteins after maturation: HA1 for binding to cell surface receptor, sialic acid; HA2 for triggering membrane fusion. Three point mutations within the receptor binding sites (a1, Y106F; a2, E199Q; a3, G237K) (21) were introduced to generate the binding-defective but fusion-competent HAmu. Single letter amino acid abbreviations are as follows: A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; K, lysine; M, methionine; P, proline; Q, glutamine; V, valine; Y, tyrosine. (*B*) FACS analysis of virus-producing cells. 293T cells were transiently transfected with plasmids encoding the following: the lentiviral vector FUGW; the membrane-bound antibody αCD20; the accessory proteins for antibodies, Igα and Igβ; the fusion protein HAmu; and viral *gag*, *pol*, and *rev* genes. Expression of αCD20 and HAmu was detected by using anti-human IgG antibody and anti-FPV HA antibody. (*C*) The class II fusion protein SINmu derived from SIN. SIN contains two membrane glycoproteins (E1 and E2) and a signal peptide (E3): E1 for mediating fusion, E2 for receptor binding, and E3 as a signal sequence for processing of E2 glycoprotein. A 10-residue tag sequence (MYPYDVPDYA) was inserted between amino acids 71 and 74 of the E2 glycoprotein. A series of alterations (a4: deletion of amino acids 61–64 of E3; a5: mutations of 68SLKQ71 into 68AAAA71; mutations of 157KE158 into 157AA158) (16) was introduced to yield the binding-defective and fusion-competent SINmu. (*D*) Directly analogous to B, except that SINmu was used for the fusion protein and was detected by an anti-tag antibody.

there exists a monoclonal antibody that allows monitoring of SIN expression; we designated it SINmu (Fig. 1*C*).

Construction of Membrane-Bound Antibody for Targeting. The antibody that we have chosen for targeting in this study is the anti-human CD20 antibody (αCD20), a version of which is currently being used in the treatment of B cell lymphomas. We generated a construct that encodes a mouse/human chimeric anti-CD20 antibody with the human membrane-bound IgG constant region (pαCD20). Genes encoding human Igα and Igβ, the two associated proteins that are required for surface expression of antibodies, were cloned into a construct designated pIgαβ.

Preparation of Recombinant Lentiviral Vectors. The production of lentiviruses enveloped with both anti-CD20 antibody and the candidate FM was achieved by cotransfection of 293T cells with the lentiviral vector FUGW, plasmids encoding viral *gag*, *pol*, and *rev* genes, pαCD20, pIgαβ and pFM (the plasmid encoding a FM, either HAmu or SINmu), by using a standard calcium phosphate precipitation method (24). FUGW is a self-inactivating and replication-incompetent lentiviral vector that carries the human ubiquitin-C promoter driving a GFP reporter gene (25). As a control, the Env derived from vesicular stomatitis virus (VSVG) was used as a joint recognition and fusion protein. FACS analysis of the transfected cells showed that virtually all expressed some level of GFP as an indicator of the presence of the viral vector (Figs. 1 *B* and *D Upper*). Some 30% of GFP-positive cells coexpressed HAmu and αCD20 on the cell surface (Fig. 1*B Lower*). A slightly smaller percentage (≈20%) of the 293T cells exhibited coexpression of GFP, SINmu, and αCD20 (Fig. 1*D*). The resultant viruses from these transfected production cells were designated FUGW/αCD20+HAmu and FUGW/αCD20+SINmu.

Coincorporation of Fusogen and Antibody into Lentiviral Vectors. To examine whether αCD20 and the FM were incorporated in the same virion, we performed a virus–cell binding assay. As a target, we made a 293T cell line stably expressing the CD20 protein antigen (293T/CD20; Fig. 2*A*). The parental cell line 293T served as a negative control. The viral supernatants were incubated with the target cells at 4°C for half an hour. The resultant binding was assayed by means of a three-staining scheme (Fig. 2*B*). FACS analysis showed that lentivectors bearing αCD20 were in fact able to bind to CD20-expressing 293T cells (Fig. 2*C Upper*). The control of 293T cells with no CD20 expression displayed no detectable αCD20, showing that the virus binding to cells must be due to a specific interaction between the cell surface CD20 antigen and the viral surface αCD20 molecule. In another control, the virus bearing only FM exhibited no ability to bind either cell line, indicating that the HAmu and SINmu did lack the capacity for cell binding (L.Y., L.B., D.B., and P.W., unpublished work). FACS analysis also showed that the virus bound to the 293T/CD20 cell surface displayed the FMs (Fig. 2*C Lower*), suggesting that both αCD20 and FM were incorporated on the same virion, which was further confirmed by FACS plots of αCD20 versus FM (Fig. 2*D*). In addition to codisplay, these results suggest that the presence of the FM does not affect the αCD20 binding to CD20.

Targeted Transduction of Lentiviral Vectors to Cell Line *in Vitro*. We next examined whether αCD20-bearing virus can transfer genes into cells expressing CD20 in a cell-specific manner. GFP expression was used to measure the transduction efficiency. The

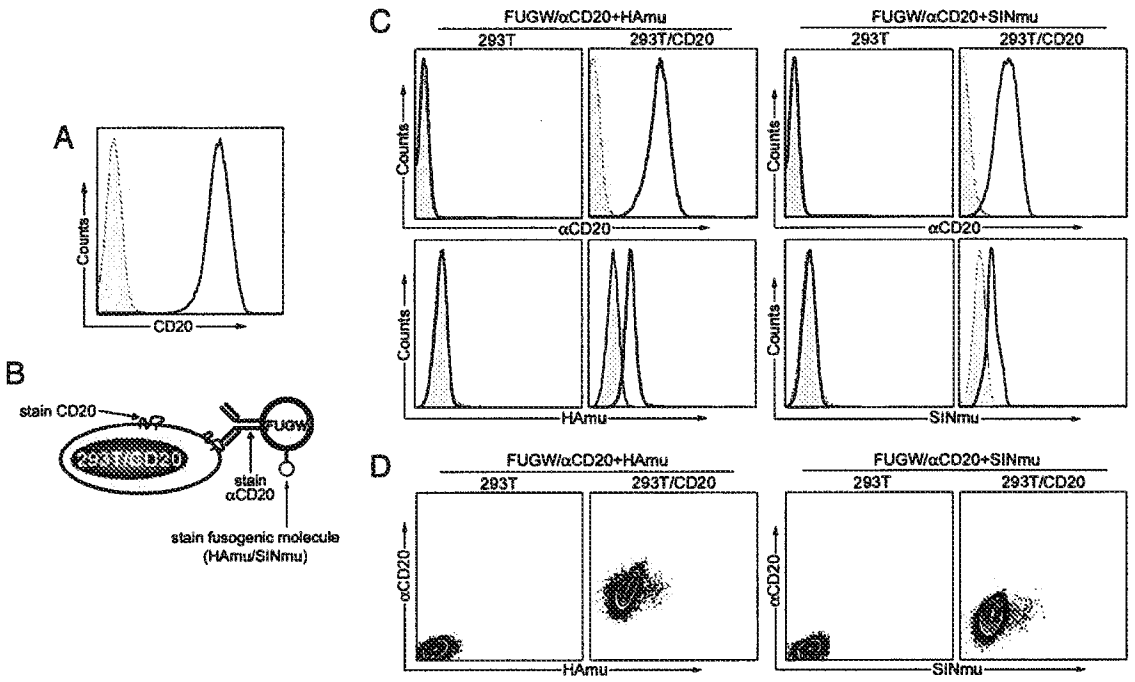

Fig. 2. Virus–cell binding assay to study the codisplay of antibody and fusogenic protein on the lentiviral surface. (*A*) FACS analysis of target cell line 293T/CD20. CD20 expression was detected by using anti-CD20 antibody. Solid line, expression of CD20 in 293T/CD20; shaded area, CD20 expression in 293T cells (as a control). (*B*) Schematic representation of three-staining scheme used for analyzing virus–cell binding assay. Three stainings were used to detect the presence of CD20, αCD20, and the fusogenic molecule (HAmu or SINmu), respectively. (*C Left*) FACS plots of 293T/CD20 cells incubated with FUGW/αCD20+HAmu. The binding of virus to 293T/CD20 cells was probed with antibody against αCD20 (anti-IgG) and HAmu. Solid line, analysis on 293T/CD20; shaded area, analysis on 293T (as a control). (*C Right*) FACS plots of 293T/CD20 cells incubated with FUGW/αCD20+SINmu. The binding of virus to 293T/CD20 cells was detected by antibody against αCD20 and SINmu. Solid line, analysis on 293T/CD20; shaded area, analysis on 293T (as a control). (*D*) Codisplay of antibody and fusogenic protein was analyzed by a density plot correlating the presence of the two proteins.

supernatants containing lentivectors bearing various surface proteins were incubated with CD20-expressing target cells, and 293T cells served as a control. Four days posttransduction, the efficiency of targeting was analyzed by FACS. Fig. 3*A* (rightmost image) shows that FUGW/αCD20+HAmu viral particles could specifically transduce 16% of 293T/CD20 cells. Images to the left show that transduction required the presence on the virions of HAmu, but there was some background transduction with virions lacking αCD20, likely because of residual weak binding of HAmu to its ligand, sialic acid. The titer for FUGW/αCD20+HAmu (fresh viral supernatant, no concentration) was estimated to be $\approx 1 \times 10^5$ transduction units (TU)/ml; the titer was determined by the percentage of GFP^+ cells in the dilution ranges that showed a linear response. The 293T cells showed a small background infection level but no specific transduction by FUGW/αCD20+HAmu (Fig. 3*A Lower*). When SINmu was used as the fusion protein, substantial enhancement of specific transduction was observed (52%; Fig. 3*B*). The titer for FUGW/αCD20+SINmu was estimated to be $\approx 1 \times 10^6$ TU/ml. Also, we detected a much lower transduction in the absence of the binding protein (≈1%). Thus, the data in Fig. 3*B* show that SINmu is a better fusion protein to partner with αCD20 for targeting lentiviral vectors. When we monitored the transduction at various time points using FACS, we found that SINmu-containing virions exhibited faster transduction kinetics than those with HAmu (L.Y., L.B., D.B., and P.W., unpublished work). Both FUGW/αCD20+HAmu and FUGW/αCD20+SINmu could be concentrated by ultracentrifugation with a >90% recovery rate, which is important for *in vivo* application.

To assess whether αCD20 and the fusion protein (HAmu or SINmu) had to be incorporated into the same viral particle, and therefore functioned in cis to mediate transduction, we mixed FUGW/αCD20 with FUGW/HAmu or FUGW/SINmu, each displaying only one protein, and tested their transduction of 293T/CD20 cells. This procedure did not result in specific transduction, indicating that the specific transduction conferred by the engineered recombinant viruses requires the two proteins to be displayed on the same viral particle.

Antibody–Antigen Interaction Responsible for Targeted Transduction. It seems that two distinct proteins can carry the binding and fusion events of engineered lentiviruses for targeted transduction. To further confirm that the specificity we observed was a consequence of interaction between αCD20 and CD20, we transduced 293T/CD20 cells in the presence of anti-CD20 blocking antibody. As expected, a dramatic decrease of infectivity was detected for both FUGW/αCD20+HAmu (L.Y., L.B., D.B., and P.W., unpublished work) and FUGW/αCD20+SINmu (Fig. 3*D*), suggesting the essential role of antibody–antigen binding for the targeted transduction.

Confirmation of pH Dependence of Fusogen. To examine the requirement for a low pH comparment to allow the recombinant lentivectors to penetrate into cells, we incubated both FUGW/αCD20+HAmu and FUGW/αCD20+SINmu with 293T/CD20 cells in the absence or presence of ammonium chloride (NH_4Cl), which can neutralize acidic endosomal compartments. Addition of NH_4Cl to cells completely abolished transduction by either FUGW/αCD20+HAmu (L.Y., L.B., D.B., and P.W., unpublished work) or FUGW/αCD20+SINmu (Fig. 3*E*). These results are consistent with the low pH requirement of HA and SIN to trigger membrane fusion. More direct evidence for pH-dependent fusion was provided by a cell–cell fusion assay. 293T cells expressing GFP,

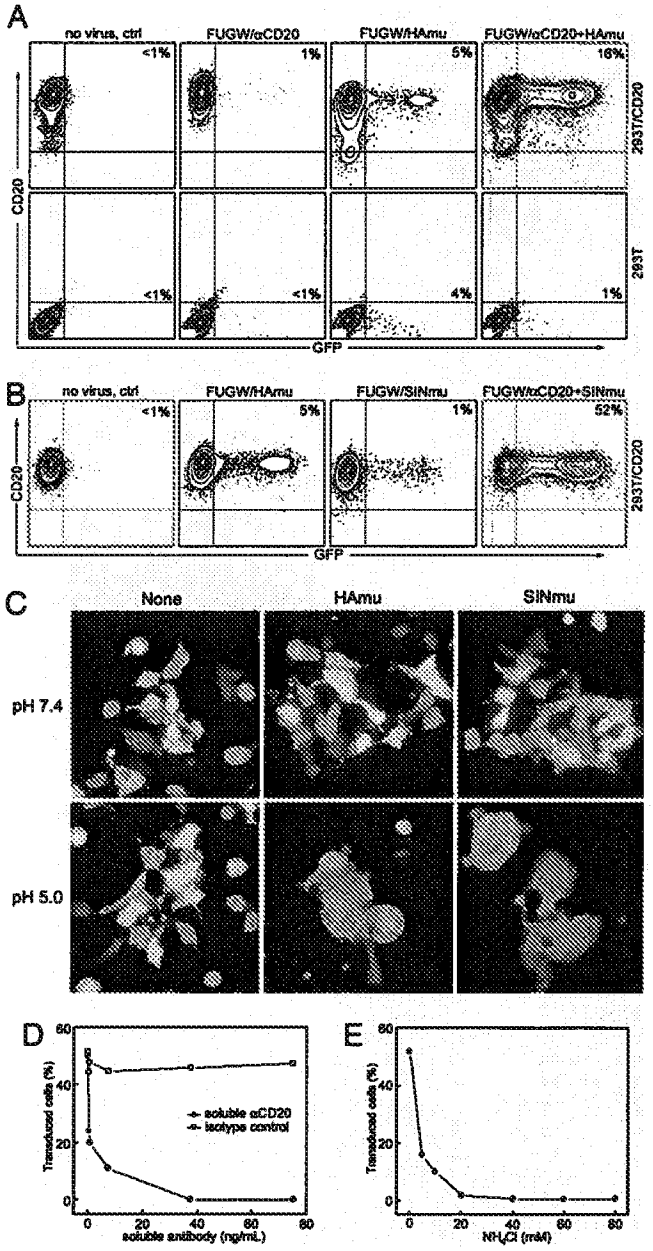

Fig. 3. Targeting of lentivectors bearing both antibody and fusion protein to 293T/CD20 cells *in vitro*. (*A*) 293T/CD20 cells (2×10^5) were transduced with 500 μl of fresh unconcentrated FUGW/αCD20 (no HAmu), FUGW/HAmu (no αCD20), or FUGW/αCD20+HAmu. 293T cells (no expression of CD20) were included as controls. The resulting GFP expression was analyzed by FACS. The specific transduction titer for FUGW/αCD20+HAmu was estimated to be $\approx 1 \times 10^5$ TU/ml. (*B*) A similar transduction experiment was performed by using unconcentrated FUGW/SINmu (no αCD20) or FUGW/αCD20+SINmu. For comparison of targeting specificity, cells were also transduced with FUGW/HAmu. The specific transduction titer for FUGW/αCD20+SINmu was estimated to be $\approx 1 \times 10^6$ TU/ml. (*C*) Evidence of pH-dependent fusion of HAmu and SINmu by a cell–cell fusion assay. 293T cells (0.1×10^6) transiently transfected to express GFP and surface αCD20 and fusion protein (either HAmu or SINmu), and 293T/CD20 cells were mixed together, washed once with normal PBS (pH 7.4), and incubated in low pH PBS (pH 5.0) or normal pH PBS (as a control) for half an hour at 37°C. The cells were then washed and cultured in the regular medium for 1 day. Cells were visualized by epifluorescence microscope equipped with a GFP filter set. (*D* and *E*) Effect of addition of soluble αCD20 (*D*) or NH_4Cl (*E*). αCD20 or NH_4Cl was added into viral supernatants during transduction for 8 h. Then, the supernatants were replaced with fresh medium. The cells were analyzed for GFP expression after 2 days. Isotype-matched antibody was used as a control for *D*.

surface αCD20, and FM were incubated with 293T/CD20 cells in a low-pH buffer for half an hour, followed by culturing in regular medium. Both HAmu and SINmu induced cell–cell fusion by forming multinucleated polykaryons (Fig. 3*C*). The interaction between αCD20 and CD20 dramatically enhances the probability of fusion, because a similar experiment with cells that lacked αCD20 and CD20 yielded a much lower level of fusion (L. Y., L.B., D.B., and P.W., unpublished work). The αCD20/CD20 interaction probably brings the cell membranes into close apposition, facilitating the action of the fusion protein.

Targeted Transduction of Lentiviral Vectors to Primary B Cells. Having established the ability of the system to mediate CD20-specific transduction of artificially created cell lines, we next investigated the possibility of specific transduction of primary human B-lymphoid cells, cells that naturally carry the CD20 antigen. Fresh, unfractionated human peripheral blood mononuclear cells (PBMCs) were transduced with FUGW/αCD20+SINmu and then stimulated with LPS to expand the B cell population. Four days later, the cells were stained for CD19 (a B cell marker), CD20, and GFP expression (Fig. 4*A*). We found that >35% of cells were $CD20^+$ B cells under our culture condition. When we gated on $CD20^+$ B cells, the majority of them were GFP^+. On the contrary, virtually no GFP^+ cells were detected when we gated on $CD20^-$ non-B cells, confirming that the transduction was strictly dependent on CD20 expression. In another control experiment, fresh PBMCs were transduced with FUGW/αCD20+SINmu followed by stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin to expand T cells. FACS analysis of these T cells showed no expression of GFP (Fig. 6, which is published as supporting information on the PNAS web site), confirming transduction specificity.

To demonstrate that the targeting method is not limited to the lentiviral vector FUGW, we evaluated two additional lentiviral vectors with different promoter configurations. Kohn and coworkers (26) have incorporated the Ig heavy chain enhancer ($E\mu$) with associated matrix attachment regions into lentivectors carrying either the human cytomegalovirus (CMV) promoter (CCMV) or the murine phosphoglycerate kinase promoter (CPGK). We adapted these two lentiviral vectors into our system and prepared recombinant lentiviruses CCMV/αCD20+SINmu and CPGK/αCD20+SINmu. Transduction of PBMC-derived B cells with these viral supernatants exhibited results similar to those observed previously with FUGW (Fig. 4*A*).

Targeted Transduction of Recombinant Lentiviral Vectors *in Vivo*. The real test of this system is whether it will mediate specific transduction *in vivo*. For this purpose, we used a human PBMC xenograft in a mouse model. Fresh human PBMCs (100×10^6 per mouse) were transferred into irradiated immunodeficient $RAG2^{-/-}\gamma_c^{-/-}$ mice through a tail vein injection. Engineered lentiviruses bearing αCD20 and SINmu were administered through the tail vein 6 h after human cell transfer. After 2 days, we collected the whole blood from these mice, and the cells were analyzed for surface antigens and GFP expression. Approximately 30–40% of the cells recovered from the mice were human T cells ($CD3^+$), and ≈0.1–0.3% were $CD20^+$ human B cells (Fig. 4*B*). Three populations were analyzed for GFP expression: $CD20^+$, $CD3^+$, and $CD20^-CD3^-$. None of the cells harvested from mice injected with virus bearing a control antibody and SINmu (FUGW/b12+SINmu) showed evidence of GFP expression in any of the three populations (Fig. 4*B*). In contrast, GFP expression was observed in at least 40% of the $CD20^+$ cells isolated from mice injected with FUGW/αCD20+SINmu whereas no transduction was detected in the other two populations.

Discussion

This demonstration of targeting efficient gene delivery vehicles strictly to the desired cell types *in vivo* greatly enhances the

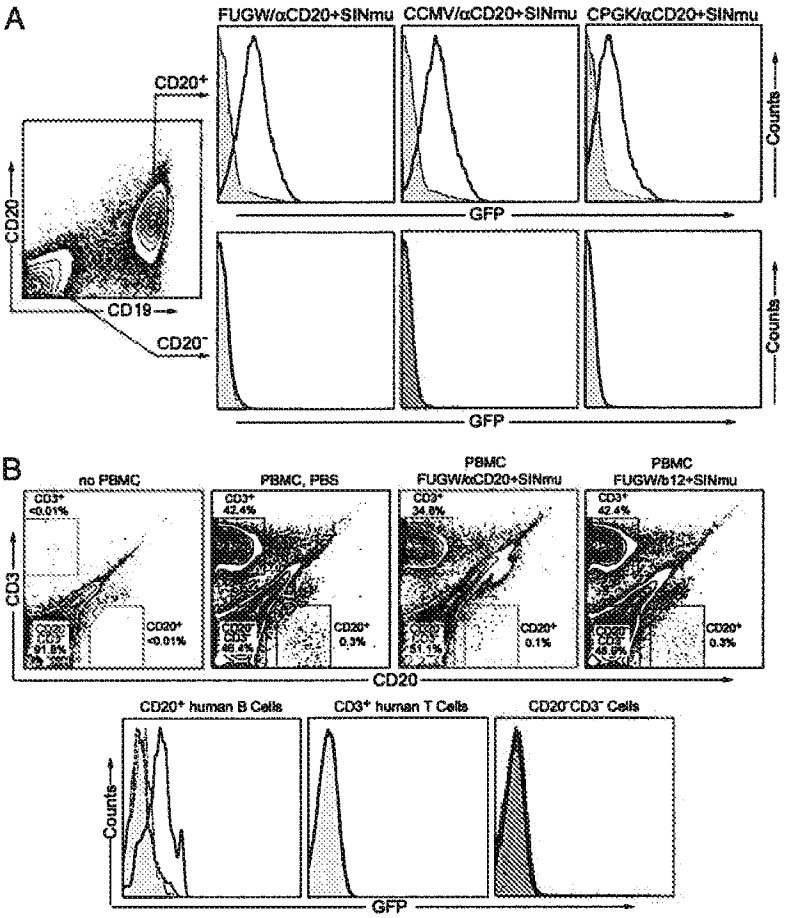

Fig. 4. Targeting CD20+ human primary B cells *in vitro* and *in vivo* using engineered lentivectors. (*A*) Fresh, unfractionated human PBMCs (2×10^6) were transduced by coculturing with concentrated FUGW/αCD20+SINmu, CCMV/αCD20+SINmu, or CPGK/αCD20+SINmu (10×10^5 TU). LPS (50 μg/ml) was added into the culture media for B cells to survive and grow. After 2 days, the B cell population was identified by costaining of CD19 and CD20. Solid line, analysis on transduced cells; shaded area, analysis on cells without transduction (as a control). (*B*) Fresh human PBMCs were transferred into irradiated RAG2$^{-/-}\gamma_c^{-/-}$ mice (100×10^6 per mouse) via tail vein injection. Six hours later, concentrated virus (100×10^6 TU per mouse) was injected through the tail vein. Two days later, whole blood was collected from these mice via heart puncture, and the cells were stained for human CD3 and CD20 and then analyzed by FACS for GFP expression. Shaded area, no virus treatment; dashed line, treated with FUGW/b12+SINmu; solid line, treated with FUGW/αCD20+SINmu.

therapeutic potential of lentivirus-mediated gene therapy and alleviates concerns of off-target effects. Possibly the most important implication of the work is that gene therapy could now be carried out as an inexpensive procedure, able to be considered even in the less-developed world.

In our approach, we break up the binding and fusion functions into two separate molecules that are inserted into the viral envelope. This methodology is particularly easy with lentiviruses (or other retroviruses) because these viruses readily incorporate into their envelope whatever proteins are found on the surface of producing cells (27). Other viruses have surfaces with many close-packed viral glycoproteins and exclude cellular proteins. A major advantage of this scheme over others where the viral protein is engineered with a foreign binding component is that the fusion protein maintains its full biological activity so that viral titer is not killed for increased specificity. The other key to the method is choosing a viral glycoprotein that mediates fusion in response to low pH and a cellular receptor that is efficiently endocytosed after antibody binding. The fusion molecule must exhibit fast enough kinetics that the viral contents can empty into the cytosol before the degradation of the viral particle. Our choice of CD20 as a target was arbitrary. We have already extended the method to other antibodies and cell surface receptor–ligand pairs.[§] We envision that the flexibility (easy combination of antibody, or other binding protein, and fusiogenic molecule) and broadness (availability of monoclonal antibodies or ligands for many endocytosed cell-specific surface molecules) of this method will facilitate the application of targeted gene delivery for therapy and research.

Materials and Methods

Construct Preparation. The cDNAs of the human κ light chain constant region and the membrane bound human IgG1 constant region were amplified and inserted downstream of human CMV and EF1αpromoters, respectively, in the pBudCE4.1 vector (Invitrogen). We cloned the light and heavy chain variable regions from the murine anti-CD20 antibody (clone 2H7) using PCR amplification and inserted them directly upstream of the corresponding constant regions. The resulting construct was designated pαCD20. We cloned cDNAs of human Igα and Igβ into the pBudCE4.1 vector (Invitrogen) to yield pIgαβ.

P. Cannon (University of Southern California and Childrens Hospital, Los Angeles) was kind enough to provide us with the construct encoding HAmu (21). We obtained the cDNA for wild-type SIN from J. Strauss's laboratory at the California Institute of Technology. PCR mutagenesis and assembly were used to generate the mutant SIN as described by Chen and colleagues (16), except a 10-residue tag sequence (MYPYDVPDYA) replaced the ZZ domain of protein A, which is located between amino acids 71 and 74 of the E2 glycoprotein of SIN. This version of SIN is designated SINmu.

Virus Production. Lentivectors were generated by transfecting 293T cells by using a standard calcium phosphate precipitation technique

[§]We have observed that this method can be exploited to target dendritic cells using a membrane-bound monoclonal antibody against the DEC-205 receptor. In addition, we found that incorporation of a membrane-bound form of stem cell factor could target c-kit-positive cells.

(24). 293T cells (≈80% confluent) in 6-cm culture dishes were transfected with the appropriate lentiviral vector plasmid (5 μg), together with 2.5 μg each of pαCD20, pIgαβ, and the packaging vector plasmids (pMDLg/pRRE and pRSV-Rev) (28). The viral supernatants were harvested 48 and 72 h after transfection and filtered through a 0.45-μm pore size filter.

To prepare high-titer lentivectors, the viral supernatants were concentrated by using ultracentrifugation (Optima L-80 K preparative ultracentrifuge, Beckman Coulter) for 90 min at 50,000 × *g*. Particles were then resuspended in an appropriate volume of cold PBS.

Cell Line Construction. The 293T/CD20 cell line was generated by stable transduction via vesicular stomatitis virus (VSVG)-pseudotyped lentivector. The cDNA of human CD20 was cloned downstream of the human ubiquitin-C promoter in the lentivector plasmid FUW to generate FUW-CD20. The lentiviral vector FUW-CD20 was then pseudotyped with VSVG and was used to transduce 293T. The resulting cells were subjected to cell sorting to obtain a uniform population of $CD20^+$ cells designated 293T/CD20.

Virus–Cell Binding Assay. Cells (293T/CD20 or 293T, 0.1×10^6) were incubated with 500 μl of viral supernatant at 4°C for half an hour and washed with 4 ml of cold PBS. The cells were then stained with the following three antibodies: an anti-human IgG antibody (BD PharMingen) to stain αCD20, an anti-human CD20 antibody (BD PharMingen) to stain CD20, and an anti-FPV HA polyclonal antibody (obtained from H.-D. Klenk, Institute of Virology, Philipps University, Marburg, Germany) to stain HAmu, or an anti-tag antibody (Roche Applied Science, Mannheim, Germany) to stain SINmu. After staining, cells were analyzed by FACS analysis.

Targeted Transduction of 293T/CD20 Cells *in Vitro*. 293T/CD20 cells (0.2×10^6 per well) or 293T cells (0.2×10^6 per well) were plated in a 24-well culture dish and spin-infected with viral supernatants (0.5 ml per well) at 2,500 rpm, 30°C for 90 min by using a Beckman Allegra 6R centrifuge. Then, the medium was removed and replaced with fresh medium and incubated for a further 3 days at 37°C with 5% CO_2. The percentage of GFP^+ cells was determined by FACS. The transduction titer was measured in dilution ranges that exhibited a linear response.

Effects of Soluble Antibody and NH_4Cl on Viral Transduction. 293T/CD20 cells (0.2×10^6) and 0.5 ml of viral supernatants were incubated for 8 h in the absence or presence of graded amounts of αCD20 (BD PharMingen) or NH_4Cl. The medium was replaced with fresh medium and incubated for another 2 days at 37°C with 5% CO_2. FACS analysis was used to quantify transduction efficiency.

Cell–Cell Fusion Assay. 293T cells (0.1×10^6), transiently transfected to express GFP, surface αCD20, and fusion protein (either HAmu or SINmu), and 293T/CD20 cells (0.1×10^6) were mixed together, washed twice with normal PBS (pH 7.4), and incubated in 150 μl of low pH PBS (pH 5.0) or normal pH PBS (pH 7.4) (as a control) for half an hour at 37°C with 5% CO_2. The cells were then washed extensively and cultured in the regular medium for 1 day. Cells were visualized by an epifluorescence microscope equipped with a GFP filter set.

Targeted Transduction of Primary Human B Cells *in Vitro*. Fresh, unfractionated human PBMCs (2×10^6) (AllCells) were incubated with concentrated virus with total TUs of 10×10^6 (based on the titer on 293T/CD20 cells). LPS (50 μg/ml) was then added for B cells to survive and grow. After 2 days, cells were harvested and washed in PBS. B cell population was determined by FACS staining using anti-human CD20 and CD19 antibodies. Targeting transduction was quantified by gating on the different populations of cells and measuring their GFP expression.

Targeted Transduction of Primary Human B Cells *in Vivo*. $RAG2^{-/-}\gamma_c^{-/-}$ female mice (Taconic) of 6–8 weeks old were given 360 rad whole-body irradiation. On the following day, 100×10^6 fresh human PBMCs (AllCells) were transferred by tail vein injection into each mouse. After 6 h, concentrated virus (100×10^6 TU per mouse) or PBS (as control) was administered via the tail vein. Two days later, whole blood was collected from these mice via heart puncture, and the cells were stained for human CD3 and CD20 and then analyzed by FACS for CD3, CD20, and GFP expression. The mice were maintained on the mixed antibiotic sulfmethoxazole and trimethoprim oral suspension (Hi-Tech Pharmacal) in a sterile environment in the California Institute of Technology animal facility in accordance with institute regulations.

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Targeted retroviral transduction of c-kit$^+$ hematopoietic cells using novel ligand display technology

Anil Chandrashekran, Myrtle Y. Gordon, and Colin Casimir

Gene therapy for a wide variety of disorders would be greatly enhanced by the development of vectors that could be targeted for gene delivery to specific populations of cells. We describe here high-efficiency targeted transduction based on a novel targeting strategy that exploits the ability of retroviruses to incorporate host cell proteins into the surface of the viral particle as they bud through the plasma membrane. Ecotropic retroviral particles produced in cells engineered to express the membrane-bound form of stem cell factor (mbSCF) transduce both human cell lines and primary cells with high efficiency in a strictly c-kit (SCF receptor)–dependent fashion. The availability of efficient targeted vectors provides a platform for the development of a new generation of therapies using in vivo gene delivery. (Blood. 2004;104: 2697-2703)

Introduction

The development of retroviral vectors that are targeted to transduce only specific cell types has been the goal of many investigators working in the field of gene therapy.[1-5] The availability of such vectors would revolutionize the field, opening up new avenues for gene therapy in acquired and inherited disorders by enabling in vivo delivery of therapeutic molecules to specific populations of target cells. Novel approaches to drug delivery and for use in vaccines also become possible through display of molecules on the retroviral surface. Over a period of around a decade, however, this goal has proven highly elusive. Virtually all attempts to target specific cell types have logically focused on modification of the retroviral envelope protein, as this complex glycoprotein is established to determine viral host range and facilitate virus entry though membrane fusion.[2,4,5] These modifications can be the simple substitution of the envelope protein for one from another retrovirus or other enveloped virus, such as vesicular stomatitis virus (VSV)[6], a process referred to as pseudotyping.[2] More recently, attempts have been made to deliberately engineer envelope proteins to redirect their binding to new cell surface molecules.[4] This has involved making N-terminal extensions, insertions, or replacements to regions of the envelope protein. Commonly used modifications have been incorporation of growth factor–binding regions[7-12] or the addition of single-chain antibodies.[13-17] While many of these modifications have successfully redirected viral binding, this has invariably been achieved at the expense of infectivity to the extent that targeted binding actually inhibited viral entry. This property has actually been used to advantage in an approach referred to as "inverse targeting."[10,18] A number of reports, however, have indicated that low levels of targeted transduction may be achievable by coexpression of an ecotropic envelope in conjunction with the modified envelope protein.[7,9,16,19]

To circumvent the problems associated with envelope modification, we have developed an alternative strategy, exploiting the natural budding mechanism of the virus to insert new binding ligands into the viral surface. We describe here successful and efficient specific targeting of transduction to human cells expressing stem cell factor (SCF) receptor (c-kit) by ecotropic retroviruses bearing surface stem cell factor.

Materials and methods

Cytokines, antibodies, and methylcellulose

All cytokines used in this study were obtained from First Link (Brierley Hill, United Kingdom), and methylcellulose used for clonogenic progenitor assays was obtained from StemCell Technologies (Vancouver, BC, Canada). Mouse anti–human c-kit antibody was obtained from R&D Systems (Abingdon, United Kingdom), mouse anti–human CD59 was from Serotec (Kidlington, United Kingdom), and anti-CD34 was from BD Bioscience (Cowley, United Kingdom). Secondary antibodies, donkey anti–goat immunoglobulin G (IgG), chicken anti–mouse IgG, and rat anti–mouse IgG were purchased from DakoCytomation Ltd (Ely, United Kingdom).

Human cell lines

The human megakaryoblastic leukemia cell line LAMA-84,[20] the monomyelocytic cell line U937, and the erythroleukemia cell line TF-1[21] were maintained in RPMI supplemented with 10% fetal calf serum (FCS). TF-1 was supplemented with 1 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF). The human megakaryoblastic leukemia cell line MO7e[22] was maintained in RPMI supplemented with 20% FCS and 10 ng/mL GM-CSF or shifted into medium without GM-CSF but containing 100 ng/mL SCF for experiments where c-kit expression was down-regulated.

Retroviral packaging and producer cell lines

Phoenix ecotropic, amphotropic (kind gifts from Dr Gary Nolan, Stanford University), and SCF-ecotropic (SCF-eco) packaging cell lines were

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routinely maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FCS (Invitrogen-Gibco, Paisley, United Kingdom). Retroviral producer lines were cultured in the same medium supplemented with puromycin (1 μg/mL) to select for the PINCO[23] retroviral genome or G418 (1.5 mg/mL) to select for pBabe-neo (pBN) and pBabe-neo–cyclin-dependent kinase 4 (CDK4).[24] The stable AM-12 retroviral producer cell line previously established in the laboratory by Chinswangwatanakul (Lewis et al[24]) was maintained in DMEM supplemented with 10% FCS and 1.5 mg/mL G418 for selection of the retroviral vector.

Construction of SCF-eco packaging cells

The membrane-bound form of stem cell factor (mbSCF) cDNA was amplified by reverse transcriptase–polymerase chain reaction (RT-PCR) from the L88.5 human stromal cell line[25] and cloned into the mammalian expression vector pREP8 (Invitrogen Ltd, Paisley, United Kingdom). Phoenix ecotropic packaging cells were transfected with pREP8-mbSCF expression vector by calcium phosphate precipitation. Packaging cells were selected for the presence of the pREP8-mbSCF plasmid by culture in histidinol. Twenty clones were obtained by culture at limiting dilution and analyzed by fluorescence-activated cell sorter (FACS) for surface SCF expression. The clone with the highest level of expression was chosen for further use.

Virus binding

MO7e cells were washed twice in phosphate-buffered saline (PBS) and incubated with retroviral supernatant (100 μL) for 1 hour at 32°C. The cells were then washed twice in cold PBS and incubated with 100 μL of anti–surface subunit (SU) monoclonal antibody, 83A25, for 1 hour at 4°C. The cells were again washed twice with cold PBS and incubated with a fluorescein isothiocyanate (FITC)–conjugated rat anti–mouse IgG antibody for 1 hour at 4°C. The cells were then washed twice in cold PBS and analyzed by flow cytometry.

CD34+ cell separation

CD34+ cells were separated from bone marrow mononuclear cells using the MiniMacs system (Miltenyi Biotec, Bisley, United Kingdom) according to manufacturer's instructions. The purity of the samples was consistently above 90%.

Estimation of retroviral titer

The pBabe vectors were titered by G418 resistance as described previously.[24] Retroviral titers of the PINCO vector in the 3 Phoenix retroviral packaging cell lines were determined on NIH3T3 cells. The cells were analyzed by flow cytometry to determine the proportion of the 3T3 cells expressing enhanced green fluorescent protein (EGFP).

Supernatant transduction of hematopoietic cells

Retroviral targeting experiments on hematopoietic cell lines were transduced using a multiplicity of infection (MOI) of 5 based on the retroviral titer on NIH3T3 cells. An infection cocktail containing retroviral supernatant, 8 mg/mL polybrene, and 10% FCS in RPMI 1640 with 2.5×10^4 target cells was incubated for 24 hours at 37°C at 5% CO_2. The medium was removed and replaced with fresh medium and incubated for a further 48 hours at 37°C at 5% CO_2. Target cells were then washed twice with PBS, fixed, and assayed by flow cytometry.

A similar protocol was followed for CD34+ cells but the transduction cocktail was supplemented with protamine sulfate (4 μg/mL), in place of polybrene, and the recombinant human cytokines fetal liver tyrosine kinase 3 (Flt-3) ligand (100 ng/mL), interleukin 3 (IL-3; 10 ng/mL), and GM-CSF (1 ng/mL). Transduction was performed for 96 hours at 37°C in Iscove modified Dulbecco medium (IMDM) medium supplemented with 30% FCS and cells were harvested and assayed as described for cell lines.

Retroviral transduction of CD34+ primary cells by Transwell coculture

The retroviral transduction protocol used in this study was previously established by Chinswangwatanakul (Lewis et al[24]), except that Flt-3 ligand was used in place of SCF. Retroviral producers were plated in a 6-well plate at a concentration of 2×10^5 cells/well and maintained in DMEM supplemented with 10% FCS. After 24 hours, the Transwell (0.4 mm; Costar, Corning Inc, Corning, NY) was inserted into each well. Immunomagnetically isolated CD34+ cells (1×10^5) in IMDM supplemented with 30% FCS, protamine sulfate (4 μg/mL), and the cytokines IL-3 (10 ng/mL), GM-CSF (1 ng/mL), and Flt-3 ligand (100 ng/mL) were then transferred to the Transwell inserts. Transduction was carried out for 48 hours before the CD34+ cells were harvested and cultured in semisolid medium, with and without G418 selection, as described below in "Granulocyte macrophage colony-forming unit (CFU-GM) assay."

Granulocyte macrophage colony-forming unit (CFU-GM) assay

The CD34+ transduced cells were cultured in 3 mL methylcellulose (Methocult H4230; StemCell Technologies) supplemented with "CFU-GM mix" (50 ng/mL SCF, 1 ng/mL GM-CSF, 10 ng/mL IL-3, and 100 ng/mL G-CSF) and 1.5 mg/mL G418. After mixing, the cells were plated into 35-mm diameter Petri dishes. The cultures were incubated at 37°C in humidified 5% CO_2. CFU-GM colonies of more than 50 cells were scored on day 14 of incubation.

PCR

DNA from transduced MO7e cells was isolated and amplified using primers located in the *EGFP* sequence. Thirty-five cycles of amplification were performed. The control amplifications were performed on the same samples using primers amplifying a common segment of the α-interferon genes. The primer sequences were identified using the software package Pride (http://pride.molgen.mpg.de). Primer sequences (EGFP-forward [F], accccgaccacatgaagcagc; EGFP-reverse [R], tcgcctcgaacttcacctc; IFN-F, gaaccagtctagcacatc; and IFN-R, ggtgagctggctacgaatc) were synthesized by Sigma-Genosys (Haverhill, United Kingdom). The products were visualized in a 2% agarose gel containing 1 μg/mL ethidium bromide.

Day-14, G418-resistant CFU-GM colonies were plucked directly into a 40-mL PCR mix containing primers specific for the *neo* resistance gene and PCR was carried out using a nested strategy as described in Lewis et al.[24] Ten microliters of the nested products were visualized, as described above.

CFU-GM replating assay

Replating assays were performed as described previously.[24] On day 7 of incubation, 90 primary CFU-GM colonies growing in the presence of G418 were plucked with a Gilson P20 pipette and replated individually into 100 mL of methyl cellulose with CFU-GM mix and 1.5 mg/mL G418 in a 96-well, flat-bottom plate (60 wells per plate with the surrounding wells filled with sterile water to prevent evaporation) for secondary colony formation. The replated cultures were allowed to continue growing at 37°C, 5% CO_2 in a humidified incubator, for a further 7 days at which point plates were scored for the presence and number of secondary colonies. The cumulative distribution of secondary CFU-GM colonies was analyzed in a logarithmic plot relating to log2n (the number of doublings required to produce n secondary colonies). The area under the curve (AUC), a parameter providing the kinetics of self-replication of progenitors, was calculated by the trapezium rule.

Results

Retroviruses are now known to acquire many intrinsic host cell membrane proteins as they bud through the plasma membrane.[26-29] We investigated whether this property could be exploited so that molecules incorporated into the retroviral envelope could be used to redirect binding and transduction of viruses to target cells

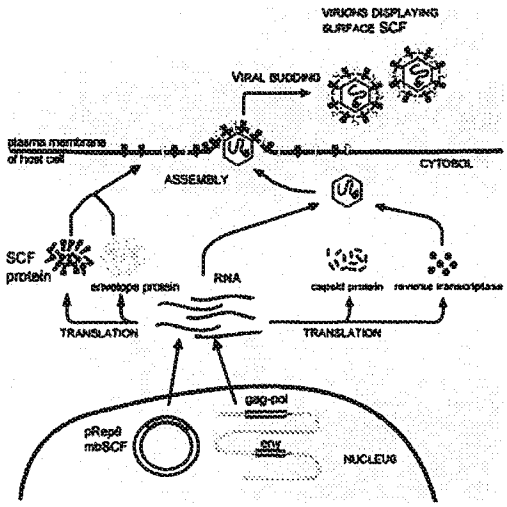

Figure 1. Schematic diagram of retroviral display methodology. Retroviral packaging cells were transfected with the plasmid pREP8-mbSCF. The cells were converted to producers by transfection with a retroviral genome. The SCF protein synthesized by these cells is expressed on the cell surface and incorporated into virus particles as they bud through the plasma membrane. Modified from Access Excellence at the National Health Museum[30] with permission.

specifically expressing the appropriate receptor (Figure 1). To this end we constructed an ecotropic retroviral producer cell line expressing the membrane-bound form of human stem cell factor (mbSCF). Using Western blotting, antibody capture of virus, and proliferation assays, we established that retroviral particles derived from these modified producers efficiently incorporated mbSCF into the retroviral envelope.[31]

Redirection of retroviral binding

Unlike ecotropic virus, which is only permissive for infection of rodent cells, virus successfully incorporating surface SCF should be capable of binding to human cells that express the SCF receptor, c-kit. We therefore performed virus-binding assays to investigate the ability of our SCF-eco viruses to bind to the c-kit–expressing cell line, MO7e.[22] As shown in the left panel of Figure 2A, anti-SU monoclonal antibody did not detect binding of normal ecotropic retrovirus to MO7e cells. Using SCF-eco virus, however, we observed a clear shift in fluorescence (Figure 2A right), indicating that the engineered virus was bound to the c-kit$^+$ target cells. To confirm that this binding was SCF mediated, the experiment was repeated using MO7e cells that had been incubated overnight with recombinant soluble human SCF. This exposure to SCF leads to a major (approximately 10-fold) down-regulation of c-kit (Figure 2B) and also reduced SCF-eco virus binding (Figure 2C right) to a level equivalent to that of unmodified ecotropic virus (Figure 2C left).

Targeted transduction of cell lines

In contrast with many retroviruses bearing modified envelope proteins, the titer of our SCF-eco virus on murine NIH3T3 cells (2.6×10^5 infectious units [ifu]/mL) was found to be comparable to those obtained with unmanipulated eco or ampho viruses (3.9×10^5 and 2.5×10^5 ifu/mL, respectively). In all subsequent experiments, volumes of retroviral supernatant were adjusted to equalize the retroviral particle numbers accordingly.

To investigate the ability of the SCF-eco virus to specifically transduce c-kit$^+$ cells, we first tested transduction of MO7e cells (Figure 2D). Efficient transduction of these cells using amphotropic (ampho) virus supernatant was observed (Figure 2Dii), whereas ecotropic (eco) virus, having a specific tropism for rodent cells, gave no evidence of transduction (Figure 2Diii). Conversely, the SCF-modified eco virus (SCF-eco) gave efficient transduction of these cells (Figure 2Div), achieving levels similar to those obtained with ampho virus. This suggested that the SCF-eco virus was capable of transducing c-kit$^+$ human cells. To extend these observations, we analyzed the relative ability of the 3 virus preparations to transduce 4 cell lines with varying levels of c-kit expression (Figure 3A): MO7e and TF-1 cells, which express high levels of c-kit, LAMA-84, which has an intermediate level, and U937, essentially c-kit$^-$. Using the high–c-kit expressers MO7e

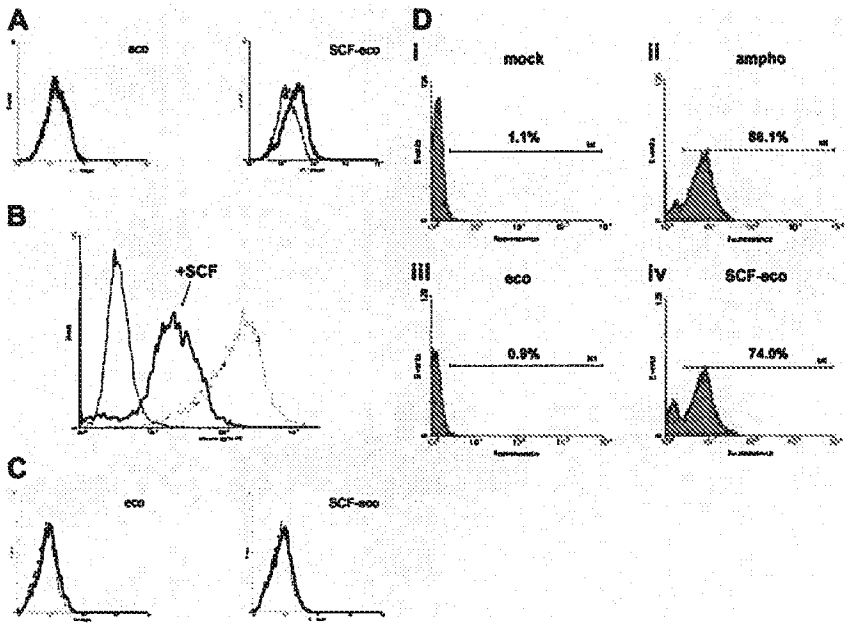

Figure 2. SCF on virus particles redirects binding and facilitates transduction of MO7e cells. (A) Virus-binding assays. MO7e cells cultured in GM-CSF were incubated with retroviral supernatants, washed, and stained for the presence of bound virus by incubation with anti-SU monoclonal antibody. The lighter trace represents cells alone and the heavy trace represents that obtained in the presence of virus. The ecotropic virus is shown in the left panel and the SCF-eco virus is shown in the right panel. (B) Down-regulation of c-kit expression in response to soluble SCF. MO7e cells were cultured in the presence of GM-CSF or for 24 hours in fresh medium containing SCF and then stained for expression of surface c-kit. The light trace is cells stained with a mouse IgG1 isotype control, the dotted trace is from cells grown in GM-CSF, and the heavy trace is from cells grown for 24 hours in SCF. (C) MO7e cells cultured for 24 hours in SCF were incubated with viral supernatant and stained with anti-SU, as described in panel A. The ecotropic virus is shown in the left panel and the SCF-eco virus is shown in the right panel. (D) Transduction of MO7e cells by ecotropic retrovirus incorporating surface SCF. Cells from the c-kit$^+$ cell line MO7e were incubated with retroviral supernatants as indicated below and transduction was evaluated by flow cytometry to detect the presence of the EGFP reporter gene. The percentage of fluorescent cells is indicated in each panel. Mock-transduced cells (i), cells transduced with amphotropic virus (ii), cells transduced with ecotropic virus (iii), and cells transduced with SCF-eco virus (iv).

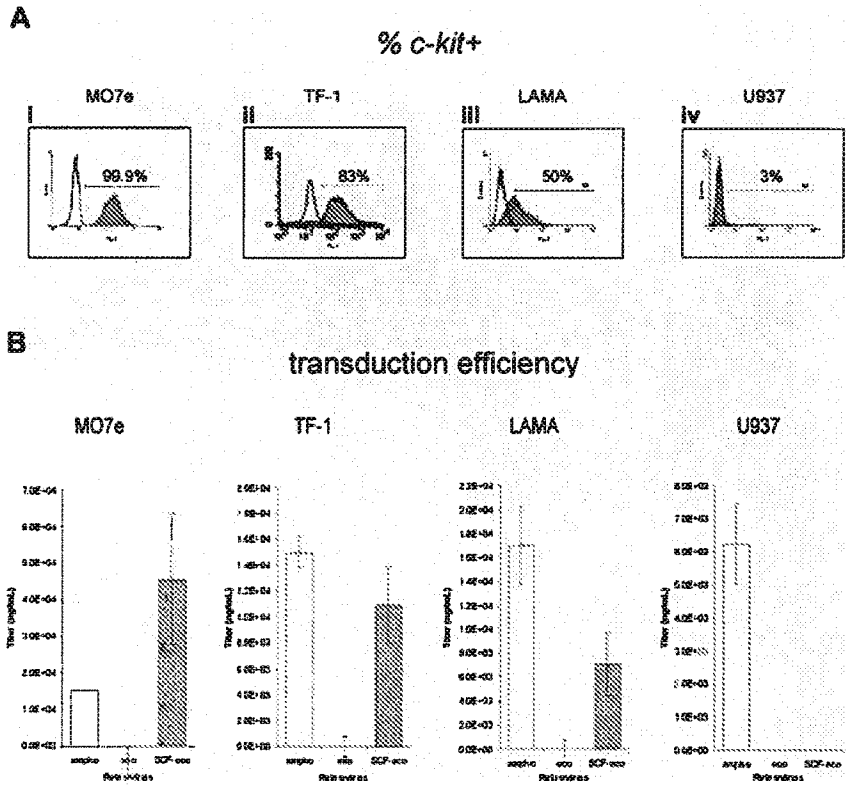

Figure 3. Transduction of human hematopoietic cell lines by SCF-eco retrovirus is c-kit dependent. (A) Staining of 4 cell lines—MO7e, TF-1 cells, LAMA-84, and U937 cells—for surface expression of c-kit. The open traces show the mouse IgG1 isotype control used for setting the fluorescence gate. (B) The 4 lines expressing different levels of c-kit were transduced (mean ± SEM) with ampho, eco, and SCF-eco retroviral supernatants as indicated. In each case, the data were obtained from 6 independent transductions and were corrected for the background level of fluorescence obtained with mock-transduced cells.

and TF-1 (Figure 3Ai-ii), the SCF-eco virus gave transduction efficiencies comparable with ampho virus (Figure 3B) and in stark contrast to the unmodified eco virus (Figure 3B), which was unable to transduce these cells. On LAMA-84 (Figure 3Aiii), the transduction by SCF-eco was significantly less efficient than before (Figure 3B), whereas on cells that do not express c-kit, the monomyelocytic cell line U937, no detectable transduction was obtained with SCF-eco or eco virus (Figure 3B). These experiments clearly demonstrated a relationship between transduction efficiency of the SCF-eco virus and the level of c-kit expression, suggesting that the SCF–c-kit interaction was mediating transduction.

To confirm the role of the virus-associated SCF in the transduction process, we repeated the transduction of MO7e cells but this time in the presence of saturating amounts of competing soluble recombinant SCF. The presence of soluble competitor virtually eradicated SCF-eco transduction of MO7e cells (Figure 4A). DNA was extracted from the transduced cell populations used in this experiment and subjected to analysis by PCR with virus-specific primers to check for the presence of the retroviral genome (Figure 4B). Consistent with the transduction data, a positive PCR signal was obtained from cells transduced with ampho or SCF-eco virus but not mock-transduced cells. Although a weaker signal resulted from cells transduced with SCF-eco virus in the presence of growth factor, the conditions used for the PCR make it unlikely that this was due to the reduced efficiency of transduction.

Targeted transduction of primary hematopoietic progenitors

We then investigated the ability of these retroviruses to transduce primary human cells. Hematopoietic progenitors were obtained from aspirates of normal bone marrow and CD34$^+$ cells isolated by

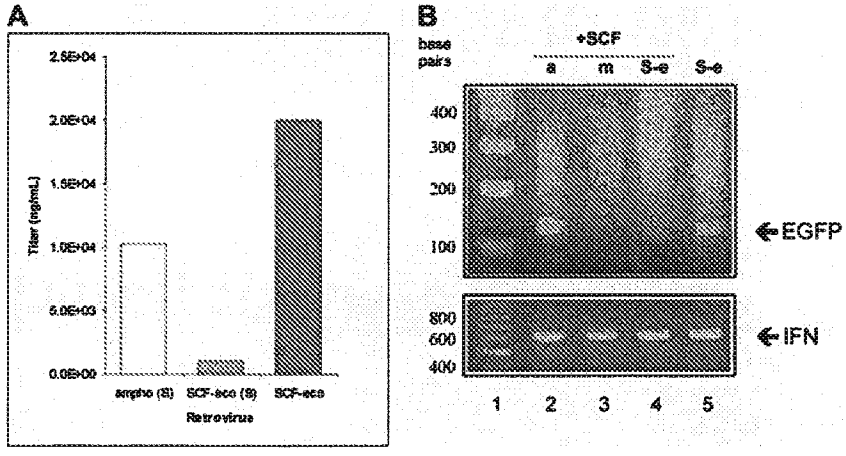

Figure 4. Transduction of a c-kit$^+$ cell line is inhibited by soluble SCF. (A) Transduction of MO7e cells, as shown in Figure 3, but performed in the presence [ampho (S) and SCF-eco (S)] or absence (SCF-eco) of 100 ng/mL human recombinant soluble SCF. Data are mean values from 2 independent transductions. (B) PCR analysis of DNA isolated from transduced cells shown in panel A. Lanes 2 to 4 show transductions performed in the presence of soluble SCF. (Top) PCR amplification using EGFP primers. (Bottom) PCR amplification of the same DNA samples using IFN-α primers. Lane 1, the 100-base-pair ladder; lane 2/a, amphotropic virus; lane 3/m, mock-transduced cells; lane 4/S-e, SCF-eco virus; and lane 5/S-e, SCF-eco virus.

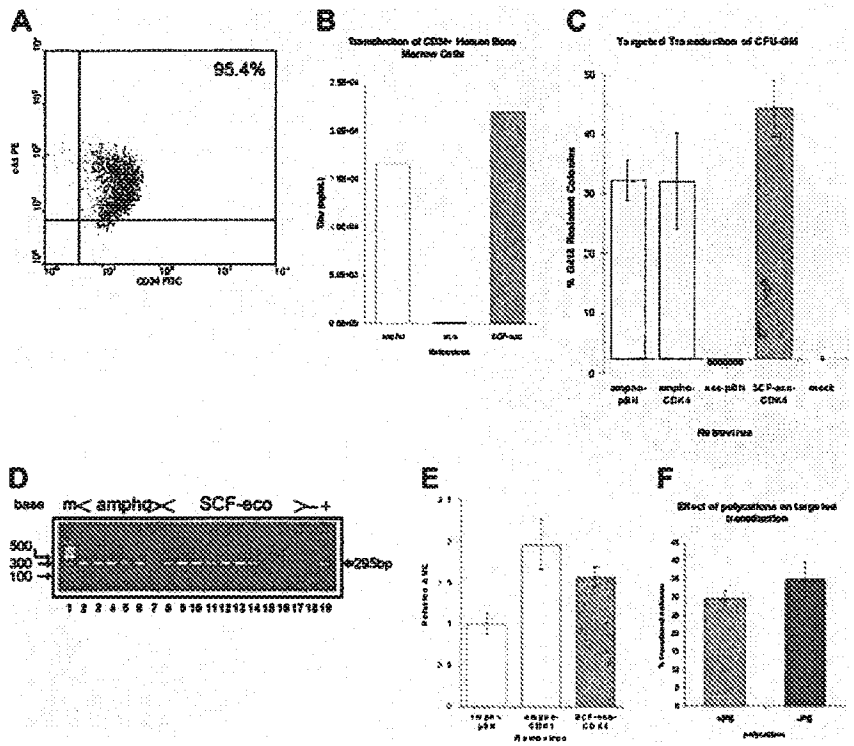

Figure 5. Efficient transduction of c-kit⁺ primary human hematopoietic cells by SCF-eco retrovirus. (A) The c-kit staining of CD34⁺ normal bone marrow. Normal human bone marrow–derived CD34⁺ cells were isolated by immunomagnetic separation and stained for CD34 with FITC-conjugated monoclonal antibody and for c-kit with a phycoerythrin (PE) conjugate. The proportion of doubly stained cells is indicated in the top right quadrant. (B) Targeted transduction of CD34⁺ bone marrow cells. CD34⁺ cells were transduced with retroviral supernatants and assayed for expression of EGFP by flow cytometry. Transduction efficiency is shown relative to the results obtained with ampho virus and data are derived as for Figure 3 but from 2 independent marrows transduced in duplicate. (C) Targeted transduction of CFU-GM. CD34⁺ cells were cocultured with retroviral producers using Transwells. The transduced cells were plated in semisolid media containing G418 to select for colonies expressing the retrovirally encoded neo gene, and resistant colonies were counted. The data (mean ± SEM) are derived from 3 independent marrows transduced in duplicate. (D) Nested PCR analysis of colonies surviving in semisolid media supplemented with G418 using primers that detect the neo gene. The figure shows data from a representative sample of the colonies analyzed. Lane 1, 100-bp ladder size marker; lanes 2-7, cells transduced with ampho virus; lanes 8-17, cells transduced with SCF-eco virus; lane18/−, semisolid media only; lane 19/+, plasmid DNA containing retroviral genome. (E) Replating assay on G418-resistant colonies. Colonies surviving in semisolid media containing G418 were replated to assay expression of the CDK4 gene encoded by the retroviral genome. Replating activity, expressed as area under the curve (AUC), was compared with that obtained from an identical retroviral vector transducing only the neo resistance marker. (F) Effect of polycations on targeted transduction. CD34⁺ progenitors were transduced as in panel C but in the presence (+PS) or absence (−PS) of 4 μg/mL protamine sulfate; transduction was determined, as before, from the percentage of colonies resistant to G418. The data shown are a representative example of the experiment that was performed 3 times.

magnetic microbead selection (MiniMacs). The CD34⁺ populations were tested for representation of c-kit and found to be greater than 90% positive (Figure 5A). Transduction of these cells with viral supernatants followed a very similar pattern to that seen previously with the cell lines. Both ampho and SCF-eco virus displayed similar transduction efficiencies, whereas the unmodified ecotropic virus gave virtually no transduction (Figure 5B).

We also tested the ability of the different viruses to transduce granulocyte macrophage colony-forming cells (GM-CFCs). For this we packaged a different vector, one that encoded a neo gene conferring resistance to the drug G418. Transductions were performed by Transwell coculture[24]; this allows prolonged exposure to target cells but prevents cell-cell contact. A typical transduction result using bone marrow–derived CD34⁺ cells is shown in Table 1 and the overall results are summarized in Figure 5C. As observed previously with this transduction system, a high proportion of G418-resistant colonies were obtained with ampho virus.[24] Nevertheless, an equivalent level of transduction was obtained using the SCF-eco virus, whereas virtually no G418-resistant colonies resulted from transduction with eco virus. To confirm that the G418-resistant colonies were truly arising from SCF-eco virus transduction of CFU-GM, some of the resistant colonies were analyzed by PCR (Figure 5D). In 15 of 20 colonies amplified using primers specific for the neo gene, a positive signal was obtained, whereas for the ampho virus, 12 of 14 colonies tested proved positive. These values are consistent (χ^2 test; $P = .34$ and $P = .72$,

Table 1. Transduction of CFU-GM from human CD34⁺ cells

	Transduction 1			Transduction 2			
Retrovirus	−G418	+G418	+G418	−G418	+G418	+G418	% resistant
Mock transduced	240	6	2	294	15	5	2.5
Ampho-pBN	77	22	21	—	—	—	27.9
Ampho-CDK4	286	28	33	170	94	—	25.4
Eco-pBN	250	2	6	—	—	—	1.6
SCF-eco-CDK4	259	93	119	227	91	94	40.8

—indicates ____.

respectively) with the frequencies of positive-scoring colonies (95%) previously observed in validating of this technique.[24] This indicated that retroviral vector genome carried in the SCF-eco retrovirus particles was present in the G418-resistant colonies.

To provide a functional test of our targeting strategy, we took advantage of the fact that the retroviral vector used in these studies also included the cDNA for the cyclin-dependent kinase, CDK4. Previous work has shown that expression of this gene in CFU-GM progenitors can increase their frequency of self-renewal in a replating assay.[24] To evaluate whether the activity of the CDK4 gene could be detected in SCF-eco–transduced colonies, some of these were replated and the replating efficiency was compared with colonies transduced with either the equivalent ampho virus or an ampho vector containing only the neo resistance marker. The colony-forming cells transduced by viruses encoding CDK4 did not differ significantly from each other in replating activity ($P = .27$), whereas cells transduced with both ampho CDK4 and SCF-eco-CDK4 showed an increased frequency of replating ($P < .05$) in comparison with those transduced with the control pBN vector, indicating activity of the CDK4 gene (Figure 5E).

In some targeting strategies, retroviral gene delivery in vitro has been found to be particularly dependent on the presence of polycations such as polybrene or lipofectamine during transduction.[32] Although it is not possible to extrapolate this to transduction in vivo,[33] reliance on such agents could compromise the ability of targeted retroviruses to deliver genes in vivo. We therefore tested the ability of our SCF-targeted viruses to deliver the neo gene to $CD34^+$ progenitors in the absence of such agents. Our standard protocols for transduction of $CD34^+$ cells include protamine sulfate. Omission of protamine sulfate from these transductions was found to have no effect on the percentage of GM colonies resistant to G418 (Figure 5F), indicating that efficient targeted transduction in our system was not dependent on the presence of polycations.

Discussion

We have described here a novel approach to retroviral targeting that exploits the natural ability of retroviruses to incorporate host cell surface proteins into the lipid envelope of the virus. By producing ecotropic virus from packaging cells that were engineered to express human membrane-bound SCF on their surface we have successfully created viruses that infect human cells with high efficiency in a strictly SCF-receptor–dependent fashion. Most significantly, we have shown this to be true not just in cell lines but also in primary cells that are potential targets for therapeutic interventions; over half the GM colony-forming cells in the $CD34^+$ population were transduced by our SCF-eco virus. Moreover, through transduction with vectors harboring the CDK4 gene, we have demonstrated that this targeting system can be used to functionally alter the properties of the targeted cells.

Many attempts have been made previously to retarget retroviral vectors to alternative cell surface receptors, including the SCF receptor,[10,34] with a view to achieving cell type specificity in target cell transduction. In essentially all cases this has been through engineering of the retroviral envelope protein itself. Unfortunately, attempts to alter the binding specificity of retroviral envelopes have nearly always compromised their ability to mediate fusion with the host cell membrane, leading to unsuccessful retroviral transduction.[5] Retroviral envelope proteins are thought to undergo major conformational changes during fusion[4] and these are most likely inhibited or hindered by addition to or altering of the envelope sequences. Targeting strategies relying on coexpression of a modified envelope and an ecotropic envelope[7,9,16,19] have provided some very limited success but, importantly, may still be severely compromised by difficulties in forming envelope trimers and in clustering of envelope proteins, steps crucial for retroviral entry.[4] This interpretation is supported by the observation that when ecotropic virus was redirected to asialoglycoprotein receptors by chemical modification with lactose, a small molecule that should not cause steric hindrance, efficient targeted transduction of a human liver cell line was demonstrated.[35]

By leaving the retroviral envelope protein intact and separating virus binding from that of fusion, our approach is able to circumvent all these problems. A similar premise has been the basis for studies in which retroviral envelope was substituted by influenza hemagglutinin (HA) to provide a fusion function.[36,37]

Regarding the precise mechanism of targeted entry in our system, the role played by the ecotropic envelope protein is of interest. It seems likely that this is providing a fusion function for viral entry, as envelope-free HIV virus–like particles (VLPs) can be made in abundance but have no infectivity, even though they are able to bind to target cells.[38] It is unclear though whether the ecotropic receptor is unusual in its apparent ability to fuse independently of binding. One clue may come from the fact that, unlike many other onco-retroviruses, ecotropic virus entry has been shown in some circumstances to be pH dependent, indicating that rather than interaction at the plasma membrane, an endosomal entry route is possible.[39] Late endosomes have a low internal pH, which could be instrumental in triggering the conformational changes required for fusion.

Endocytosis would be an advantageous route of entry, as late endosomes are primarily perinuclear[40]; this may differ from amphotropic viruses that are pH-independent for entry. This difference in entry route of targeted and amphotropic viruses might explain the somewhat reduced level of reporter gene expression we observed with targeted transduction. This may result in lower copy number transductions or, perhaps, access to different sites of nuclear integration leading to lower levels of expression.

Targeted vectors in particular would be released from the necessity to perform transductions ex vivo and could herald a new phase of development of gene therapy protocols using in vivo gene delivery. This would be particularly felt in the arena of acquired disease but also has major ramifications for treating inherited disorders.

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